Exhibit 17

Transcriptomic and Proteomic Analysis of Steatohepatitic Hepatocellular Carcinoma Reveals Novel Distinct Biologic Features

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ABSTRACT

Objectives: *Steatohepatitic hepatocellular carcinoma is a distinct variant of hepatocellular carcinoma strongly associated with underlying nonalcoholic steatohepatitis. The molecular biology of steatohepatitic hepatocellular carcinoma is not fully elucidated, and thus we aimed to investigate the molecular underpinnings of this entity.*

Methods: *Transcriptomic analysis using RNAseq was performed on eight tumor-nonneoplastic pairs of steatohepatitic hepatocellular carcinoma with comparison to conventional hepatocellular carcinoma transcriptomes curated in The Cancer Genome Atlas. Immunohistochemistry was used to validate key RNA-level findings.*

Results: *Steatohepatitic hepatocellular carcinoma demonstrated a distinctive differential gene expression profile compared with The Cancer Genome Atlas curated conventional hepatocellular carcinomas (n = 360 cases), indicating the distinctive steatohepatitic hepatocellular carcinoma morphology is associated with a unique gene expression profile. Pathway analysis comparing tumor-nonneoplastic pairs revealed significant upregulation of the hedgehog pathway based on* GLI1 *overexpression and significant downregulation of* carnitine palmitoyltransferase 2 *transcript. Glutamine synthetase transcript was significantly upregulated, and fatty acid binding protein 1 transcript was significantly downregulated and immunohistochemically confirmed, indicating steatohepatitic hepatocellular carcinoma tumor cells display a zone 3 phenotype.*

Conclusions: *Steatohepatitic hepatocellular carcinoma demonstrates a distinctive morphology and gene expression profile, phenotype of zone 3 hepatocytes, and activation of the hedgehog pathway and repression of* carnitine palmitoyltransferase 2*, which may be important in tumorigenesis.*

Key Points

- Steatohepatitic hepatocellular carcinoma displays a zone 3 phenotype based on upregulated glutamine synthetase and downregulation of fatty acid binding protein 1 both at the RNA and protein levels.
- Steatohepatitic hepatocellular carcinoma demonstrates a distinctive differential gene expression profile compared with conventional hepatocellular carcinoma, which corresponds to a unique morphology.
- Hedgehog pathway activation based on *GLI1* overexpression and significant downregulation of *carnitine palmitoyltransferase 2* transcript may be contributors to the tumor biology in steatohepatitic hepatocellular carcinoma.

Nonalcoholic fatty liver disease is a condition characterized by hepatic steatosis with or without steatohepatitis and fibrosis, which is associated with the metabolic syndrome consisting of obesity, insulin resistance, hypertension, and hypercholesterolemia.[1-3] The subset with nonalcoholic steatohepatitis has the potential to show progressive fibrosis and evolve to cirrhosis. Due to the global obesity pandemic, the prevalence of nonalcoholic fatty liver disease and nonalcoholic steatohepatitis has been constantly increasing.[4]

A variant of hepatocellular carcinoma, termed *steatohepatitic hepatocellular carcinoma*, was described by Salomao et al[5] in 2010. This subtype demonstrates

histomorphologic features similar to steatohepatitis within the neoplastic hepatocytes, including steatosis, hepatocyte ballooning, Mallory-Denk bodies, pericellular fibrosis, and inflammation. Studies have identified an association between steatohepatitic hepatocellular carcinoma and nonalcoholic fatty liver disease/nonalcoholic steatohepatitis, which may be the first morphologic subtype of hepatocellular carcinoma to be linked with an etiology.[5,6] As the prevalence of nonalcoholic steatohepatitis increases, the prevalence of steatohepatitic hepatocellular carcinoma is likely to rise as well, reinforcing the clinical importance of better comprehension of this entity. Notably, a minority of steatohepatitic hepatocellular carcinoma is not associated with nonalcoholic fatty liver disease/nonalcoholic steatohepatitis, possibly developing the steatohepatitic histomorphology due to having similar genetic changes.[7] Thus, understanding the biology of steatohepatitic hepatocellular carcinoma may have broader relevance.

To date, little is known about steatohepatitic hepatocellular carcinoma carcinogenesis and its molecular characteristics. Elucidation of the molecular alterations involved in steatohepatitic hepatocellular carcinoma could be exploited to provide novel treatment options for patients. Our goal is to determine if the distinct histomorphology of steatohepatitic hepatocellular carcinoma corresponds to a distinctive gene expression profile and identify recurrent pathway and genetic alterations.

Materials and Methods

Case Selection

After internal review board approval at each author's institution, the authors' case files (T.M., R.K.M., M.O., D.S.A., A.M.B., S.M.L., and R.P.G.) were reviewed for cases of steatohepatitic hepatocellular carcinoma diagnosed on liver explant or partial hepatectomy specimens between 2010 and 2018. Steatohepatitic hepatocellular carcinomas diagnosed on needle core biopsy were excluded, and cases that received chemoembolization prior to surgical resection were included. All slides and formalin-fixed, paraffin-embedded tissue blocks were procured. H&E- and immunohistochemical-stained slides for each case were reviewed by the authors at their respective institutions to confirm steatohepatitic hepatocellular carcinoma diagnosis of the tumor nodule consistent with the definition determined by Salomao et al,[6] including the presence of three or more of the following features in the tumor nodule: steatosis, neoplastic hepatocyte ballooning, Mallory-Denk bodies, pericellular fibrosis, and inflammation. However, our study required a threshold of more than 95% steatohepatitic features in the tumor nodule compared with the 50% or more threshold set by Salomao et al.[6] If multiple nodules of hepatocellular carcinoma, some of which may not be steatohepatitic hepatocellular carcinoma, were present in one explant or partial hepatectomy specimen, only one steatohepatitic hepatocellular carcinoma nodule was sufficient for the diagnosis of steatohepatitic hepatocellular carcinoma and included in the study if it also met the inclusion criteria listed above. If multiple steatohepatitic hepatocellular carcinoma nodules were present that met the inclusion criteria in the same case, one representative nodule was selected for inclusion. No more than one steatohepatitic hepatocellular carcinoma tumor nodule per patient was included in the study. All steatohepatitic hepatocellular carcinoma cases were re-reviewed at Mayo Clinic by four of the authors (B.J.V.T., T.M., R.K.M., and R.P.G.) to ensure each case met the inclusion criteria. In total, 19 cases were identified that met the inclusion criteria. Of the 19 cases, 11 were from Mayo Clinic, and the other eight cases were from other institutions. The eight external cases made up the external cohort of steatohepatitic hepatocellular carcinomas and were used as an external validation cohort for immunohistochemical analysis of steatohepatitic hepatocellular carcinomas as described below in the methods section on immunohistochemistry.

A chart review was performed to record the following clinicopathologic data from each case: age, sex, etiology of liver disease, α-fetoprotein level (ng/mL), total number of hepatocellular carcinoma nodules per liver specimen, number of steatohepatitic hepatocellular carcinoma nodules per specimen, size of the steatohepatitic hepatocellular carcinoma nodule included in the study, presence of background steatohepatitis, histologic grade, and pathologic T stage.

Transcriptomic Analysis

Of the 19 total steatohepatitic hepatocellular carcinoma cases, 11 cases from Mayo Clinic were selected for whole transcriptome analysis using RNAseq methodology as previously described.[8] Formalin-fixed, paraffin-embedded tissue sections of steatohepatitic hepatocellular carcinoma tumor and paired nonneoplastic tissue from the same 11 cases were used for transcriptome analysis. Three of the 11 cases selected for RNAseq failed, leaving a total of eight steatohepatitic hepatocellular carcinoma cases analyzed via RNAseq successfully. The data generated were analyzed with ENSEMBL annotation (ensembl.

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org) and Ingenuity Pathway Analysis (Qiagen). Gene expression profile analysis was performed comparing differential gene expression of the eight steatohepatitic hepatocellular carcinoma tumor-nonneoplastic tissue pairs compared with 360 hepatocellular carcinoma transcriptomes curated in The Cancer Genome Atlas. Further gene expression profile analysis using The Cancer Genome Atlas hepatocellular carcinoma transcriptomes with documentation of associated chronic liver disease (hepatitis C, n = 35; hepatitis B, n = 23; alcoholism, n = 62) was also examined. SVA and ComBat statistical methods were used to correct for potential batch effects.[9,10]

Molecular classification of steatohepatitic hepatocellular carcinoma based on hepatic lobule zonal gene expression compared with nonneoplastic tissue was evaluated using the following genes: argininosuccinate 1 (*ASS-1*), fatty acid binding protein 1 (*FABP1*), and glutamine synthetase (*GLUL*).

The following carcinogenic pathways' gene expression in the tumor compared with nonneoplastic tissue was evaluated: carnitine palmitoyltransferase 2 expression because of its role in fatty acid metabolism, sonic hedgehog pathway via *GLI1* expression because of its role in steatohepatitis and the mammalian target of rapamycin (mTOR), and nuclear factor–κB (NF- κ B), interleukin (IL)–6/JAK/STAT, and WNT/β-catenin pathways because they have been proposed to be altered in hepatocellular carcinoma.[11-19]

Immunohistochemistry

Immunohistochemistry with commercially available antibodies was performed at Mayo Clinic on formalin-fixed, paraffin-embedded tissue sections of the steatohepatitic hepatocellular carcinoma tumor nodule selected from each case for the following proteins: liver fatty acid binding protein (1:500 dilution; Santa Cruz Biotechnology), glutamine synthetase (1:2,000 dilution; Millipore), and β-catenin (prediluted; Ventana). The tissue blocks were sectioned at 4 μm, deparaffinized, and processed on the Ventana BenchMarkXT immunohistochemical stainer (Ventana, Roche Diagnostics). Following processing, the tissue sections were counterstained with hematoxylin.

The expression of liver fatty acid binding protein and glutamine synthetase from steatohepatitic hepatocellular carcinoma tissue sections was quantitated and compared with adjacent nonneoplastic hepatocytes by calculating an H-score, with scores ranging from 0 to 300, for both the neoplastic and nonneoplastic tissue from all 19 cases, with eight of the 19 cases constituting an independent external (non–Mayo Clinic cases) validation cohort. The H-score was calculated using previously reported methods by multiplying the staining intensity (0 = none, 1 = weak, 2 = moderate, 3 = strong) with the percentage of cells staining unequivocally positive.[20] β-Catenin was assessed only with the Mayo Clinic cohort (n = 11) by evaluating the nuclear staining intensity as either positive (nuclear staining) or negative (no nuclear staining). All immunohistochemical slides were examined by two pathologists (B.J.V.T. and R.P.G.), and any discrepancies were resolved by consensus.

Statistical Analysis

Transcriptome statistical analysis was performed using IBM SPSS Statistics 25. Wilcoxon signed rank test was applied in the statistical analysis of liver fatty acid binding protein and glutamine synthetase staining intensity. *P* values less than or equal to .05 were considered statistically significant.

Results

Patient and Case Characteristics

All 19 steatohepatitic hepatocellular carcinoma specimens, including the independent external validation cohort (n = 8), met the morphologic inclusion criteria as shown in Image 1 and were from explant hepatectomy or partial hepatectomy specimens from patients with a median age of 64 years (range, 35-75 years). There was a male predominance, with a male/female ratio of 16:3. Ten (53%) cases had nonalcoholic steatohepatitis alone as the underlying chronic liver disease. The other nine cases were not associated with nonalcoholic fatty liver disease/nonalcoholic steatohepatitis or metabolic syndrome per clinical notes and had viral hepatitis C alone (n = 3), viral hepatitis C with alcoholic liver disease (n = 1), viral hepatitis C with hereditary hemochromatosis (n = 1), viral hepatitis B (n = 1), or idiopathic (n = 1) as the etiology underlying chronic liver disease. One case had steatohepatitic hepatocellular carcinoma diagnosed incidentally in the background of primary sclerosing cholangitis and cholangiocarcinoma, and another patient had sarcoidosis. Most cases (n = 12) were well differentiated, with the remaining being moderately differentiated (n = 7). The median number of hepatocellular carcinoma nodules per case was two with a range from one to seven. The number of steatohepatitic hepatocellular carcinoma nodules per case ranged from one to six nodules per case with a median of one steatohepatitic hepatocellular carcinoma nodule per case. The size of the steatohepatitic hepatocellular carcinoma nodule randomly selected for inclusion in the study from each case ranged in size from 0.9 to 4.6 cm, with a median size of 2.3 cm. The median α

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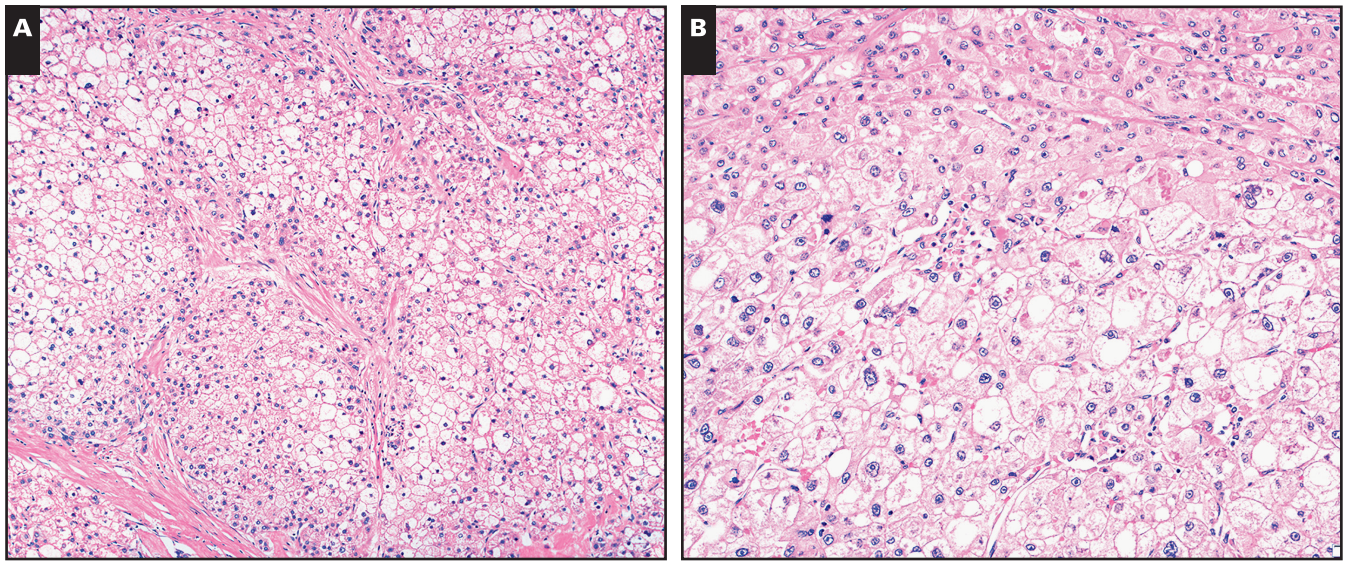

Image 1 All 19 steatohepatitic hepatocellular carcinoma cases met the inclusion criteria, which included more than 95% of the tumor possessing three or more of the following histologic characteristics: steatosis, neoplastic hepatocyte ballooning, Mallory-Denk bodies, inflammation, and pericellular fibrosis (**A**, H&E, ×100; **B**, H&E, ×200).

-fetoprotein level was not elevated prior to liver transplantation with a value of 6.85 ng/mL (range, 1.6 to 3,726 ng/mL). Pathologic T staging and all clinicopathologic characteristics for the combined cohort (all Mayo and external cases), Mayo Clinic cohort, and external cohort are listed in Table 1.

Steatohepatitic Hepatocellular Carcinoma Displays Diminished Zone 1 and Increased Zone 3 Markers

Multiple studies have elucidated the gene expression profiles of the zones of the hepatic lobule. These profiles correlate with the active metabolic processes present in each respective zone.[21-24] For example, periportal or zone 1 hepatocytes are responsible for arginine metabolism, the urea cycle, and fatty acid oxidation. Thus, genes associated with these processes, such as argininosuccinate 1 and fatty acid binding protein 1, are upregulated in zone 1 hepatocytes.[25] Pericentral or zone 3 hepatocytes participate in nitrogen metabolism, among their many other functions, and require glutamine synthetase expression. We investigated the hepatic lobule gene expression profiles via RNAseq to ascertain the zonation profile of eight steatohepatitic hepatocellular carcinomas. Comparison of the steatohepatitic hepatocellular carcinoma transcriptomes to corresponding nonneoplastic liver revealed upregulation of *glutamine synthetase* (fold change, 1.33; $P = .019$) and downregulation of *fatty acid binding protein 1* (fold change = 0.53; $P = .00046$) and *argininosuccinate 1* (fold change = 0.33; $P = 9.1 \times 10^{-17}$). These results were validated at the protein level using immunohistochemistry, which showed significantly decreased staining intensity for liver fatty acid binding protein (nonneoplastic median H-score = 255, tumor median H-score = 120, Wilcoxon signed rank test, $P = .003$) and increased staining intensity for glutamine synthetase (nonneoplastic median H-score = 60, tumor median H-score = 180, Wilcoxon signed rank test, $P = .005$) Image 2. An independent external (non–Mayo Clinic cases) validation cohort consisting of eight cases, from two external institutions, further confirmed the results for liver fatty acid binding protein (nonneoplastic median H-score = 210, tumor median H-score = 145, Wilcoxon signed rank test, $P = .05$) and glutamine synthetase (nonneoplastic median H-score = 65, tumor median H-score = 215, Wilcoxon signed rank test, $P = .011$) Table 2. Taken together, the tumor cells in steatohepatitic hepatocellular carcinoma show an expression pattern in keeping with zone 3 hepatocytes.

Steatohepatitic Hepatocellular Carcinoma Possesses a Distinct Gene Expression Profile Compared With Other Hepatocellular Carcinomas

Differential gene expression analysis of the eight paired steatohepatitic hepatocellular carcinoma tumor/nonneoplastic tissues compared with 360 The Cancer Genome Atlas hepatocellular carcinoma transcriptomes revealed clustering of the eight steatohepatitic hepatocellular carcinoma cases together distinct from conventional hepatocellular carcinoma, shown in Figure 1A. To mitigate potential batch effects of comparing two different transcriptomic sequencing experiments (ours and The Cancer Genome Atlas), we used SVA and ComBat

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Table 1
Clinicopathologic Characteristics of the Steatohepatitic-Hepatocellular Carcinoma Cases

Characteristic	Combined Cohort (n = 19)	Mayo Clinic Cohort (n = 11)	External Cohort (n = 8)
Age, median (range), y	64 (35-75)	60 (35-69)	68 (55-75)
Male/female, No.	16/3	9/2	7/1
No. of cases with multiple nodules of HCC	11	8	3
No. of HCC nodules per case, median (range)	2 (1-7)	2 (1-7)	1 (1-3)
No. of SH-HCC nodules per case, median (range)	1 (1-6)	1 (1-6)	1 (1-1)
SH-HCC nodule size included in the study, median (range), cm	2.3 (0.9-4.6)	1.6 (0.9-3.3)	3 (2.7-4.6)
No. of cases following neoadjuvant therapy	5	3	2
α-Fetoprotein level, median (range), ng/mL	6.85 (1.6-3,726)[a]	5.1 (1.6-78)	11 (2.5-3,726)[a]
Chronic liver disease etiology, No.			
NASH	10	6	4
Hepatitis C	3	3	0
Hepatitis C and alcohol	1	0	1
Hepatitis C and hemachromatosis	1	0	1
Hepatitis B	1	1	0
Sarcoidosis	1	0	1
PSC and cholangiocarcinoma	1	1	0
Idiopathic	1	0	1
Histologic grade, No.			
Well differentiated	12	4	8
Moderately differentiated	7	7	0
Poorly differentiated	0	0	0
Undifferentiated	0	0	0
Pathologic T stage,[b] No.			
pT1	1	0	1
pT1a	3	3	0
pT1b	2	1	1
pT2	12	7	5
pT3	1	0	1
pT4	0	0	0

HCC, hepatocellular carcinoma; NASH, nonalcoholic steatohepatitis; PSC, primary sclerosing cholangitis; SH-HCC, steatohepatitic hepatocellular carcinoma.
[a]α-Fetoprotein level for one case was unavailable.
[b]Based on American Joint Committee on Cancer eighth edition.

corrected analysis to compare the gene expression profiles. We included only the transcriptomes with a curated chronic liver disease etiology (viral hepatitis C, n = 35; viral hepatitis B, n = 23; and alcoholism, n = 62) and a nonsteatohepatitic hepatocellular carcinoma morphology as reviewed by the authors. This second gene expression profile analysis confirmed steatohepatitic hepatocellular carcinoma clustered in two separate groups that did not include any other cases from The Cancer Genome Atlas on their respective terminal branch points **Figure 1B**. The smaller group included three patients who received pretransplant chemoembolization (with >90% tumor viability) in contrast to the other five patients. Thus, the unique steatohepatitic hepatocellular carcinoma histomorphology may be the result of a distinct gene expression profile.

Hepatocarcinogenic Pathway Analysis

The sonic hedgehog pathway showed significant activation across all cases while the following pathways were not significantly or recurrently up- or downregulated across all cases: NF-κB, mTOR, IL-6/JAK/STAT, and WNT/β-catenin.

The sonic hedgehog pathway appeared to be significantly activated, as evidenced by upregulation of a key downstream target *GLI1* (fold change = 2.2, P = .047, false discovery rate = 0.22). For all of the other examined pathways (NF- κ B, mTOR, IL-6/JAK/STAT, and WNT/β-catenin), we found some of the downstream gene targets upregulated and others downregulated compared with nonneoplastic hepatic tissue without an overall statistically significant activation or repression of the pathways (data not shown).

Given that diffuse glutamine synthetase expression may be related to activation of the WNT/β-catenin pathway, we not only looked for transcriptomic evidence of activation of this pathway but also performed β-catenin immunohistochemistry. The WNT/β-catenin pathway was found to be downregulated, but this was not statistically significant (P > .05). When the *β-catenin* transcript expression in steatohepatitic hepatocellular carcinoma was compared with nonneoplastic tissue, there was

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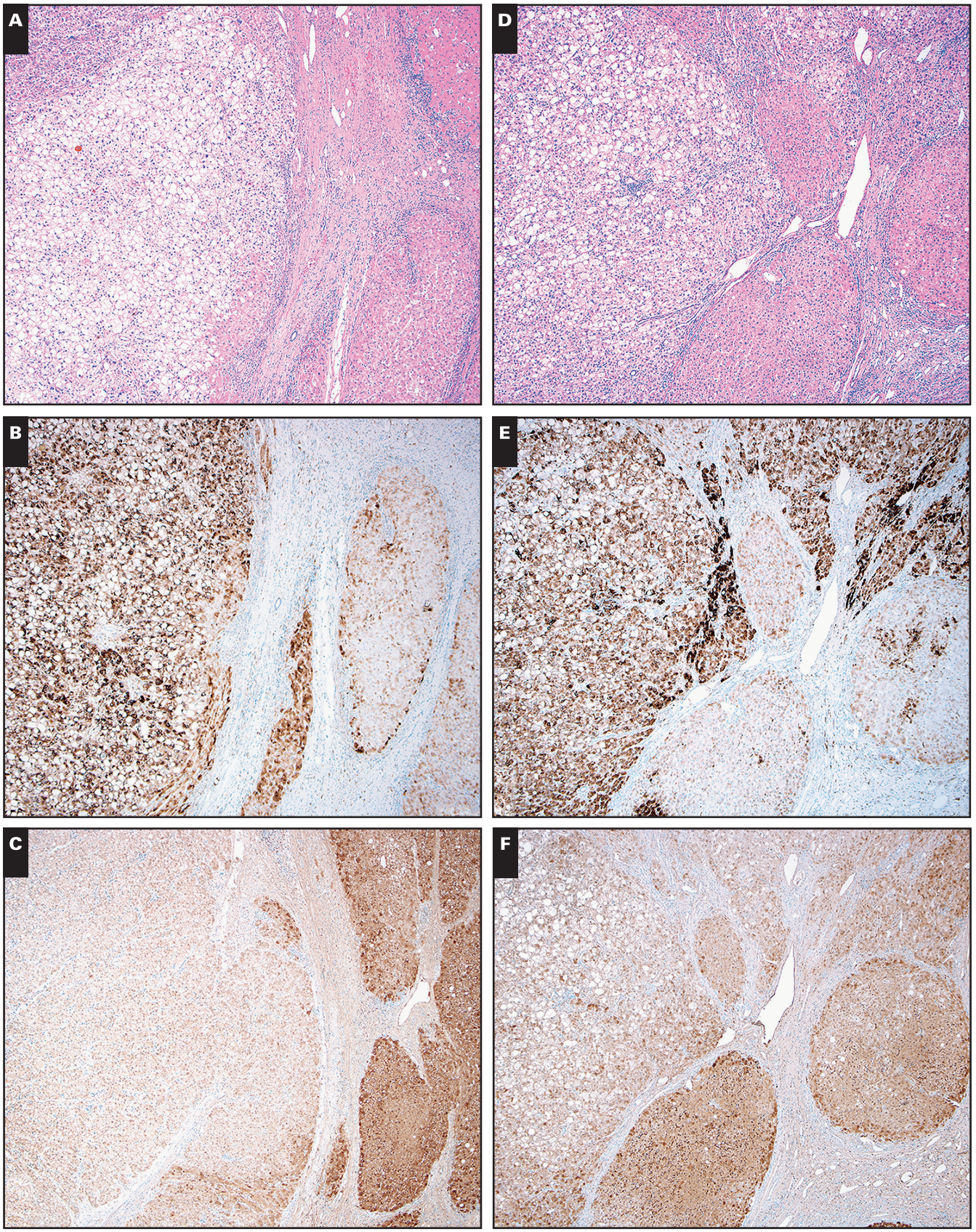

Image 2 Steatohepatitic hepatocellular carcinoma displays a zone 3 gene and immunohistochemical expression profile with two representative examples depicted (**A**, **D**, H&E, ×100) with respective immunostains listed below each H&E image. Steatohepatitic hepatocellular carcinoma displays reduced staining intensity for liver fatty acid binding protein (**C**, **F**, ×100) and increased staining intensity for glutamine synthetase (**B**, **E**, ×100) compared with adjacent nonneoplastic liver on the right side of the images.

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Table 2

LFABP and GS Staining Intensity for Mayo Clinic Cohort and an Independent External Validation Cohort

	LFABP H-Scores			GS H-Scores		
Characteristic	**Nonneoplastic**	**Neoplastic**	***P* Value**	**Nonneoplastic**	**Neoplastic**	***P* Value**
Mayo Clinic cohort (n = 11)	255	120	.003	60	180	.005
Independent external validation cohort (n = 8)	210	145	.05	65	215	.011

GS, glutamine synthetase; LFABP, liver fatty acid binding protein.

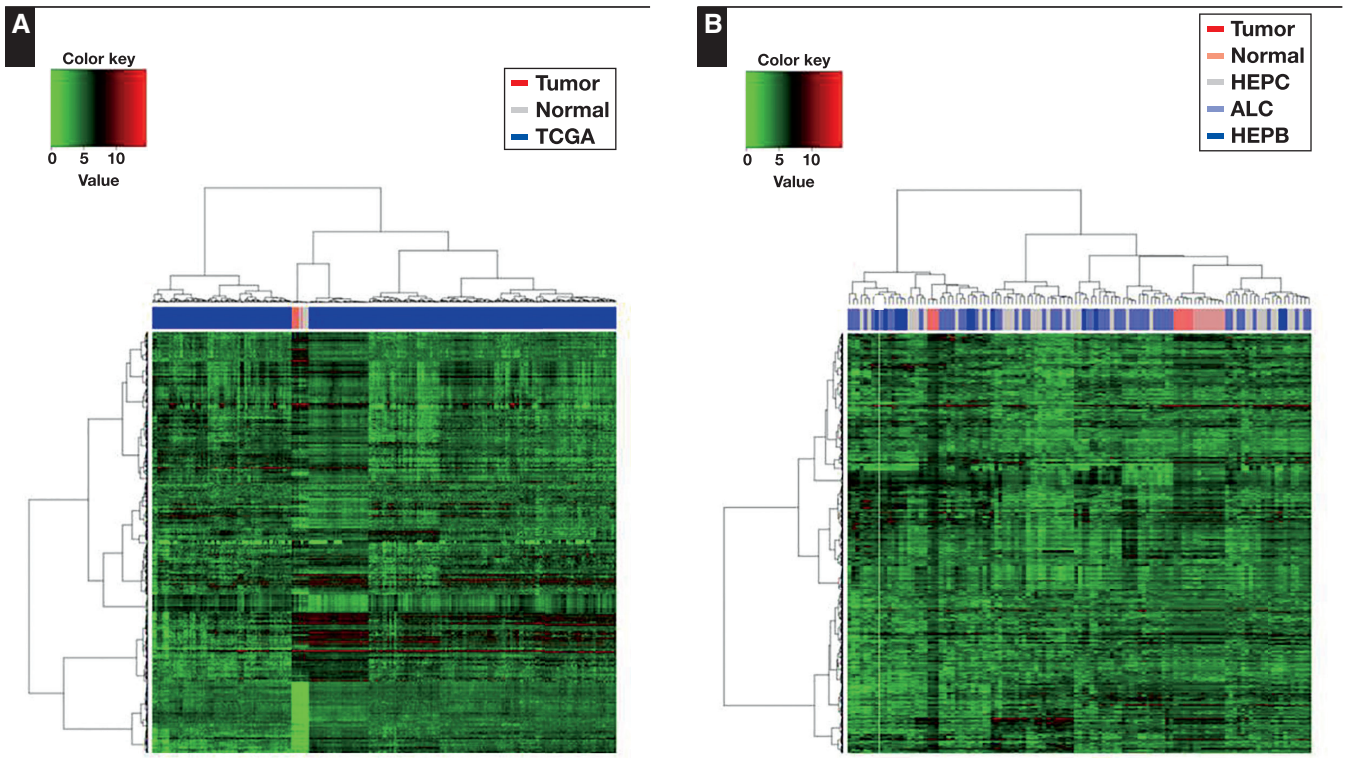

Figure 1 **A**, Heatmap diagram comparing the differential gene expression of eight steatohepatitic hepatocellular carcinoma cases with 360 cases of hepatocellular carcinomas from The Cancer Genome Atlas (TCGA). The heatmaps demonstrate a common gene expression profile among the steatohepatitic hepatocellular carcinoma cases compared with other cases in TCGA. **B**, Heatmap diagram comparing the differential gene expression of eight steatohepatitic hepatocellular carcinomas and adjacent nonneoplastic liver tissue (designated as tumor and normal, respectively) and curated cases from TCGA of hepatocellular carcinoma without steatohepatitic morphology, with known chronic liver disease etiology (viral hepatitis C [HEPC], n = 35; viral hepatitis B [HEPB], n = 23; and alcoholism [ALC], n = 62) corrected for batch effect with SVA and ComBat. Steatohepatitic hepatocellular carcinoma cases occupy their own final branch in two groups. The smaller group received chemoembolization in contrast to the other group.

no significant difference (fold change = 1.04, false discovery rate = 0.91, P = .77). Immunohistochemistry for β-catenin validated this finding, as none of our 11 cases tested by immunohistochemistry demonstrated nuclear staining.

In addition, we found decreased expression of *carnitine palmitoyltransferase 2* in steatohepatitic hepatocellular carcinoma (fold change = –0.7201, false discovery rate = 0.012, P = .00072).

Discussion

Steatohepatitic hepatocellular carcinoma is a morphologically distinctive variant of hepatocellular carcinoma. Our results show several novel findings. The distinctive morphology of steatohepatitic hepatocellular carcinoma corresponds to a distinctive gene expression profile that differs from conventional hepatocellular carcinoma in two separate analyses. In our second analysis,

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which used bioinformatic tools to correct for potential batch effects, we noted that the cohort of steatohepatitic hepatocellular carcinoma was separated into two groups of cases. The smaller group included cases treated with pretransplant chemoembolization. While these tumors were more than 90% viable, it is possible that the effects of chemoembolization influenced the tumor biology and separated this subgroup.

Multiple prior studies have shown hedgehog pathway activation in active steatohepatitis for reparative purposes.[12-15] Our data show that the hedgehog pathway is further upregulated based on *GLI1* overexpression against a background of mildly active steatohepatitis (seen in the background liver in eight cases evaluated with RNAseq). These findings suggest that the hedgehog pathway is involved in the preneoplastic injury and also in the progression of steatohepatitis.[12-15] Therefore, the hedgehog pathway may be an important targetable pathway for treatment of patients with steatohepatitic hepatocellular carcinoma. Our study is the first to provide RNA-level evidence of hedgehog pathway activation in steatohepatitic hepatocellular carcinoma and is in accord with two others studies that provided protein-level evidence of hedgehog pathway overexpression in steatohepatitic hepatocellular carcinoma compared with conventional hepatocellular carcinoma by sonic hedgehog ligand immunohistochemistry in tissue microarrays.[20,26] Along with hedgehog activation in steatohepatitis, studies have identified the epicenter of steatohepatitis injury typically occurs in zone 3 of the hepatic lobule, and zone 3 is the first zone to histologically demonstrate fat accumulation in nonalcoholic fatty liver disease.[27] Interestingly, our transcriptomic and immunohistochemistry data support the conclusion that the neoplastic hepatocytes in steatohepatitic hepatocellular carcinoma exhibit a zone 3 expression pattern characterized by significant downregulation of fatty acid binding protein 1 and argininosuccinate synthase 1, as well as upregulation of glutamine synthetase at both RNA and protein levels. This extends the proposal of considering hepatocellular neoplasia based on zonation as reported in other studies.[24,28,29] Along those lines, this may open up a paradigm of differential targeting of hepatocellular neoplasms based on their metabolic profiles.

Overexpression of glutamine synthetase often is associated with β-catenin dysregulation, as glutamine synthetase expression is partially controlled by the WNT/β-catenin pathway.[30,31] Interestingly, upregulation of β-catenin was not seen at the RNA level, and there was no nuclear accumulation of β-catenin at the protein level. These findings are in line with those of Ando et al,[20] who reported that nuclear β-catenin accumulation and *CTNNB1* mutations are rare in steatohepatitic hepatocellular carcinoma compared with conventional hepatocellular carcinoma. The authors included both cases with pure steatohepatitic hepatocellular carcinoma morphology and mixed steatohepatitic hepatocellular carcinoma and conventional hepatocellular carcinoma in their group of steatohepatitic hepatocellular carcinoma, and so it is not clear if the rare cases of steatohepatitic hepatocellular carcinoma with β-catenin expression in their series were those with pure steatohepatitic hepatocellular carcinoma morphology. Calderaro et al[32] concurred with our observations and did not identify *CTNNB1* mutations or WNT/β-catenin pathway upregulation in steatohepatitic hepatocellular carcinoma. Taken together, these data indicate that glutamine synthetase upregulation in steatohepatitic hepatocellular carcinoma is probably influenced by another pathway.

Another important novel finding in our study is diminished expression of carnitine palmitoyltransferase 2 in steatohepatitic hepatocellular carcinoma seen in all of our cases. Carnitine palmitoyltransferase 2 is a mitochondrial enzyme with an essential role in fatty acid β-oxidation and carnitine metabolism. Carnitine palmitoyltransferase 2 is responsible for conversion of fatty acids to acyl-coenzyme A and carnitine.[11] High levels of fatty acids are a feature of liver tumors in the obese,[33] and such levels may lead to increased fatty acid oxidation and JNK-mediated hepatocyte death.[11] However, in vitro studies showed that decreased levels of carnitine palmitoyltransferase 2 protect neoplastic hepatocytes from lipotoxicity.[11] Therefore, reduced levels of carnitine palmitoyltransferase 2 are believed to facilitate neoplastic cell survival in obesity-related hepatocellular carcinoma. Concordant with this notion, low levels of carnitine palmitoyltransferase 2 have been identified in hepatocellular carcinoma tissue from mice and humans with obesity-related hepatocellular carcinoma.[11] Notably, evidence of decreased carnitine palmitoyltransferase 2 was seen in hepatocellular carcinoma in nonobese mice, but the degree of reduction was significantly less than obesity-related hepatocellular carcinoma.[11] This suggests that while this metabolic change happens broadly in hepatocellular carcinoma, it is accentuated and potentially more important in hepatocellular carcinoma in obesity models.[34] None of the preceding studies were focused on the morphology of the hepatocellular carcinoma beyond the presence of steatosis. Our study emphasized careful morphologic assessment for study inclusion and identified consistent downregulation of carnitine palmitoyltransferase 2, suggesting that carnitine palmitoyltransferase 2 downregulation may be an adaptive mechanism in steatohepatitic hepatocellular

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carcinoma, provides evidence that fatty acid oxidation metabolism is altered in steatohepatitic hepatocellular carcinoma, and raises the possibility that fatty acid metabolism may be a tractable target in steatohepatitic hepatocellular carcinoma.

Our study is limited by not comparing steatohepatitic hepatocellular carcinoma with other variants of hepatocellular carcinoma in the same experiments. Therefore, we compared it with the publicly available The Cancer Genome Atlas data set and employed statistical tools to mitigate the bias of comparing different transcriptomic data sets. An important strength of this study design is the strict definition of steatohepatitic hepatocellular carcinoma.

In summary, steatohepatitic hepatocellular carcinoma's unique morphologic appearance corresponds to a distinct gene expression profile compared with conventional hepatocellular carcinoma. Furthermore, steatohepatitic hepatocellular carcinoma appears to recapitulate zone 3 hepatocytes, both at the transcriptomic and protein levels, extending a paradigm of a metabolic-focused classification of hepatocellular neoplasia. We also describe several key observations with regard to the molecular biology of steatohepatitic hepatocellular carcinoma by identifying activation of sonic hedgehog pathway and significant downregulation of *carnitine palmitoyltransferase 2*.

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